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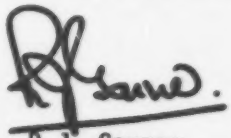
FOREWORD

The U.S. Environmental Protection Agency was created in response to increasing public concern about the dangers of pollution of the health and welfare of the American people and their environment. The complexities of environmental problems require an integrated program of research and development using input from a number of disciplines.

The Health Effects Research Laboratory was established to provide sound health effects data in support of the regulatory activities of the EPA. A key segment of such a data bank is the knowledge of the effects of a pollutant directly on a cell. Cell culture studies provide fundamental data on the potential mutagenicity, fibrogenicity and carcinogenicity of materials tested.

This report presents the results of a study to determine the relative levels of cytotoxicity and mutagenicity for the different varieties of asbestos fibers. An understanding of the effects of asbestos on the cellular level is important in determining the potential health effects of asbestos in drinking water.



R.J. Garner
Director
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ABSTRACT

Three forms of Union Internationale Contre le Cancer (UICC) asbestos: amosite, crocidolite and chrysotile, were assayed for their cytotoxicity (inhibition of colony formation) and mutagenicity (induction of mutants at the hypoxanthine-guanine phosphoribosyl transferase [HGPRT] locus) in cell culture. Using embryonic human intestine derived (I-407) and adult rat liver-derived (ARL-6) epithelial cells, the order of toxicity was chrysotile > amosite ≈ crocidolite. All three asbestos types were more toxic to I-407 than to ARL-6 cells. Asbestos was also tested for inhibition of colony formation in cultures of mouse colon-derived epithelial-like cells (MCE-1); the toxic effects produced in these cells were similar to those in I-407 cells. Leaching the asbestos for three days in sterile deionized water did not appreciably affect the toxicity of the three asbestos forms. Leaching in hydrochloric acid, however, slightly increased the toxicity of amosite and crocidolite and greatly decreased the toxicity of chrysotile. During leaching in deionized water, substantial levels of Mg^{++} and Ca^{++} were released from the asbestos fibers into the leaching fluid. Greater titers of these ions were released during leaching in hydrochloric acid.

Mutagenesis assays, utilizing the toxic purine analog 6-thioguanine for selection of HGPRT deficient mutants, revealed that high concentrations of chrysotile, crocidolite or amosite were not mutagenic in cultures of rat liver-derived epithelial cells.

Samples of contamination from six municipal water supplies were also tested for cytotoxicity by measuring the inhibition of colony formation in I-407 cultures. The toxicity of these samples was much less than that of equivalent amounts of standard asbestos materials.

This report was submitted in fulfillment of Grant No. R-803998010 by the Naylor Dana Institute for Disease Prevention, American Health Foundation, under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from January, 1976 to December, 1978 and was completed as of January 29, 1979.

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ABBREVIATIONS

ARL-6	Adult rat liver-derived epithelial cells (transformed)
CFE	Colony forming efficiency
EPA	U.S. Environmental Protection Agency
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
I-407	Embryonic human intestine-derived epithelial cells
MCE-1	Mouse descending colon-derived epithelial-like cells
UICC	Union Internationale Contre le Cancer

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SECTION 1

INTRODUCTION

The five main varieties of asbestos that are of economic importance to various countries are chrysotile, crocidolite, amosite, anthophyllite and tremolite. In the United States where over 800,000 tons of asbestos are consumed annually, chrysotile is the form of asbestos in greatest commercial use (94%). Crocidolite (4.4%), amosite (1.1%) and anthophyllite (0.1%) also have commerical significance. Natural asbestos deposits occur worldwide, but in the United States, asbestos is mined and milled primarily at five locations in California (70%), Arizona and Vermont. However, most of the asbestos consumed in the United States is imported (90%); chrysotile is imported primarily from Canada (96%) and the Republic of South Africa (3%). (12, 24)

Asbestos fibers can enter the environment from natural geological deposits, particularly during mining and milling. In the United States, there are five mining and milling sites; of these, only one mine is underground while four are surface mines which are responsible for maximal escape of asbestos fibers into the atmosphere. Asbestos is also released during the manufacture of end-products, shipment, usage and disposal. The mining of ores geologically associated with asbestos, such as talc and iron, is another major source of environmental contamination with asbestos. (12,24)

Human exposure to asbestos can occur as a result of any of these industry-related activites or from the use of asbestos-containing products. Inspired asbestos fibers are found in the respiratory system, and in addition, these fibers can migrate across internal membranes and have been detected in thoracic and abdominal lymph nodes, liver, spleen, pancreas and kidney (13, 22). Fibers also infiltrate the gastrointestinal tract via pulmonary clearance (11) and as the result of ingesting asbestos-contaminated water and certain commercially prepared beverages, foods and drugs (7, 8, 30, 32, 39). Data indicate that asbestos penetrates the gastrointestinal mucosa (48, 51-53, 32, 42) and produces biochemical changes in the rat small intestine mucosa and lumen (18).

Asbestos is of grave concern as a health hazard because of its cytotoxic, fibrogenic and carcinogenic properties (24). Exposure to this mineral is associated with an increased

incidence of bronchogenic carcinomata and pleural and peritoneal mesotheliomata in humans (3, 35). An increased incidence of cancers of the gastro-intestinal tract in asbestos workers has also been reported (36).

The objectives of this investigation were to develop and utilize *in vitro* techniques with a variety of cell types for the delineation of the biological effects of asbestos fibers, particularly as they relate to cancer induction in the gastrointestinal tract. Part of this objective consisted in developing cytotoxicity and mutagenicity assays. The cytotoxicity of three forms of asbestos was examined in human embryonic intestine-derived epithelial cells (I-407), adult rat liver- derived epithelial cells (ARL-6) and mouse descending colon mucosal epithelial-like cells (MCE-1). Mutagenicity assays were performed on ARL-6 cultures, only. As a corollary to these studies, samples of asbestos were leached in water or hydrochloric acid, and the cytotoxicities of leached samples of amosite, crocidolite and chrysotile were compared with the toxicities of unleached material. Samples of solid material isolated from municipal water supplies, provided by the U.S. Environmental Protection Agency (EPA), were also assayed for their cytotoxicity.

SECTION 2

CONCLUSIONS

Three forms of Union Internationale Contre le Cancer (UICC) asbestos: amosite, crocidolite and chrysotile were analyzed for their cytotoxicity and mutagenicity using assay systems developed in this laboratory. When assayed for their effect on the colony forming efficiency of human embryonic intestine-derived I-407 and rat liver-derived ARL-6 epithelial cells, all three forms of asbestos produced marked cytotoxicity. Chrysotile was approximately ten-fold more toxic than the amphiboles. All three asbestos types were more toxic to I-407 than ARL-6 cells. Chrysotile was found to have a similar toxic effect on MCE-1 and I-407 cells; this suggests that intestinal epithelial cells may be of particularly high sensitivity to asbestos. The reproducibility of the results from the cytotoxicity assay demonstrates the usefulness of assaying for the inhibition of colony formation as a reliable method for the quantitation of cytotoxicity. This assay measured the cytotoxicities of samples isolated from six different municipal water supplies and, therefore, appears useful for screening environmental samples.

UICC amosite, crocidolite and chrysotile were leached for three days in sterile, deionized water. This procedure did not appreciably affect the toxicity of the different asbestos forms, although analysis of the leachates for Mg^{++} and Ca^{++} content by atomic absorption spectrophotometry revealed that extraction of Mg^{++} and Ca^{++} had occurred. Leaching in 5N HCl greatly decreased the toxicity of chrysotile, while slightly increasing the toxicity of amosite and crocidolite. Atomic absorption spectrophotometry demonstrated a sizeable loss of Mg^{++} and Ca^{++} from all three asbestos fibers, particularly chrysotile, due to 5N HCl-leaching. These results suggest a convergence of toxicity of chrysotile and the amphiboles during prolonged emersion in a fluid *in-vitro*; a similar process may occur *in vivo*. This phenomenon could explain why the two classes of asbestos fibers, although different in their short-term effects, are not clearly different in their long-term effects, i.e., delayed cytotoxicity (25).

Assays for mutagenesis at the HGPRT locus failed to demonstrate a consistant or significant increase in the number of mutants resistant to 6-thioguanine after exposure

to crocidolite, chrysotile or amosite. The lack of significant mutagenicity that can be attributed to asbestos in these experiments is consistent with the concept that asbestos is not a genotoxic carcinogen characterized by interaction with DNA but rather is an epigenetic carcinogen of the solid state type (54). Solid state carcinogens appear to assert their carcinogenic effects through physical interactions with cells. An assay that measures physical interactions could therefore be a reliable method for the quantitation of carcinogenicity of a solid state carcinogen. If the physical changes produced by asbestos are responsible for its cytotoxicity, then the cytotoxicity assay used in this study may be a suitable assay for the quantitation of potential carcinogenicity of asbestoses as well as their cytotoxicity.

From the above results, it is concluded that chrysotile and crocidolite are potentially the most hazardous forms of asbestos. Furthermore, specific cell types, such as those of intestinal origin, may be particularly sensitive to the toxic effects of asbestos.

SECTION 3

RECOMMENDATIONS

The results of our studies suggest the following recommendations:

First, further work should be undertaken concerning the physical and chemical properties of the various asbestos fibers and the biological effects of altering these properties. As can be seen from the leaching data in Section 6, the chemical and physical properties of asbestos are readily altered and it is relatively easy, in the laboratory, to produce changes in these properties that have great biological significance. Leaching can also occur *in vivo* (19, 25) or even geologically. In this regard, Langer (21) reports that asbestos fibers found in older sediments of Lake Superior show evidence of iron loss due to leaching and differ significantly in composition from freshly mined fibers. Thus, although asbestos is relatively heat-stable, asbestos fibers are subject to change by means of leaching, in the laboratory or in a biological or geological environment. Our studies and those of others (16, 19, 25, 26, 28, 55) demonstrate that this process may increase or decrease asbestos reactivity, depending upon the type of asbestos leached and the leaching conditions. Therefore, to insure the safe handling of asbestos and to further elucidate the toxic and carcinogenic characteristics of asbestos, a thorough understanding of the physical and chemical properties and the biological effects of altering these properties is required. Such information should be made readily available to laboratory investigators and epidemiologists conducting asbestos-related studies, as well as safety control officers in asbestos plants.

Secondly, detailed studies on the mechanism(s) of the carcinogenic action of asbestos are important. Our studies and the work of others (6,17) indicate that asbestos is not a genotoxic carcinogen. Furthermore, removal of the surface ligands from asbestos fibers by leaching in water or acid appears to influence the cytotoxic (25, 26, 31) as well as the tumorigenic (28) potential of asbestos fibers. Thus, mechanisms of action have been proposed to explain asbestos carcinogenicity which emphasize the reaction of surface ligands, such as Mg^{++}, of phagocytosed asbestos fibers with lysosomal membrane sialoglycoproteins (9, 14, 15, 43, 44). Such mechanisms, however, have not been confirmed nor do they

include the ultimate production of tumors. It is, therefore, necessary for specific reactive groups on asbestos fibers to be identified and the effects of these groups on cells to be elucidated. Related phenomena, such as the biological coating of asbestos with protective proteins (10), also require explanation. Once the basic mechanisms of asbestos carcinogenicity are understood, it is possible that means for the reduction or even elimination of asbestos carcinogenicity can be devised.

Thirdly, as an extension of the second recommendation, further work should be undertaken concerning the biological effects of asbestos *in vivo* and *in vitro*, particularly as it affects gastro-intestinal cells. Selikoff (36) reported an excess of gastro-intestinal cancer in asbestos workers; however, it is a more complex task to establish an increased incidence that can be specifically attributed to asbestos that is ingested during occupational exposure or from the consumption of contaminated drinking water, medication, or commercial food and beverages. Thus, *in vivo* and *in vitro* cell and organ culture studies are needed to establish gastro-intestinal cancer as a direct result of ingested asbestos. Furthermore, recent studies (33, 40) indicate that pleural and peritoneal tumors are produced by implanted fibers of a specific size, regardless of composition; it would be of interest to verify these experiments in the gastro-intestinal tract, as well as to determine whether ingested fibers can pass through the walls of the digestive tract and produce tumors in more distal organs. Also, the results in Section 6 of this study indicating that two cell lines of intestinal origin are more sensitive to asbestos than a liver-derived line deserve confirmation and extension.

Finally, efficient methods for the bioassay of asbestos-induced effects should be developed. In order to more effectively study the biological and epidemiological effects of asbestos, it is imperative that practical methods be developed for the identification and quantification not only of amounts but also of the biological effects of asbestos. The cytotoxicity assay used in this investigation provides a rapid and reliable means for quantitating the inhibitory effect of asbestos on colony formation of mammalian cells. The results of this assay are a measure of the physical alteration produced by asbestos in cells and may be further used to evaluate asbestos as a solid state carcinogen.

SECTION 4

MATERIALS AND METHODS

Asbestos Samples

The UICC standard reference samples provided by the EPA were Canadian chrysotile B, crocidolite and amosite (45). Before weighing, samples of asbestos were exposed to 130 ergs/mm^2/sec ultraviolet light for 2 hours in order to suppress viable contamination in a manner that would be least likely to alter the physical or chemical properties of the asbestos fibers. In order to eliminate inaccuracies in weighing caused by the electrostatic character of asbestos, aliquots of asbestos were weighed in a known weight of water or culture medium immediately prior to use.

Cell Cultures

All cells were maintained at 37°C in Williams' Medium E (Flow Laboratories, Rockville, Maryland) supplemented with 10 percent fetal bovine serum (Flow Laboratories) and containing 5.0 units/ml mycostatin (Gibco, Grand Island, New York) and 100 µg/ml gentamycin (Schering, Kenilworth, New Jersey).

I-407 and ARL-6 cells were used for most of the studies in this project. MCE-1 cells, isolated and maintained in this laboratory, but, as yet, not fully characterized, were also used for cytotoxicity assays.

For the isolation of the MCE-1 cells, the descending colon mucosa of 6-12 week-old neomycin and mycostatin-pretreated mice was digested with 0.25 percent pronase and then scraped gently to detach the cells. These cells were pelleted at 50xg for four minutes and washed twice. Yields of 1.5 x 10^6 viable cells (85 to 95 per cent trypan blue negative) per cm colon were obtained. These cells were a mixture of epithelial and fibroblast-like cells. Collagenase (50 units/-ml) was used to enrich the primary cultures with epithelial cells. When the primary cultures were at least 80 per cent confluent, they were trypsinized and transferred. One of the clones developed from these cultures was the MCE-1 cell line.

Mg++ and Ca++ Determinations

Atomic absorption spectrophotometry was utilized for the

determination of Mg^{++} and Ca^{++} concentrations in the post-leaching supernatants (leachates). Both filtered and unfiltered aliqots of leachate were analyzed.

Mutagenesis Assay

The measurement of mutations at the HGPRT locus was examined using a protocol described previously (46), as follows:

1. Twenty-four hours after exposure to asbestos, exposed and control cells were trypsinized, counted, and reseeded separately at 20 cells/cm^2 in 25cm^2 flasks for colony forming efficiency (CFE) determination and at 1.3 x 10^3 cells/cm^2 in 75 cm^2 flasks for maintenance during mutation expression time.
2. Six days later, the cells seeded at 20 cells/cm^2 were fixed in formalin and stained for CFE determination.
3. After four and nine days, control and asbestos-treated cells, seeded in step 1, were trypsinized and reseeded separately at cell concentrations permitting optimal growth (i.e. cell division occurring every 15 to 17 hours).
4. After fourteen days, control and treated cultures were seeded for CFE determinations as in Step 1 and at 10^4 cells/ cm^2 in 25 cm^2 flasks for selection of HGPRT deficient mutants.
5. Replacement of the culture medium with analog (10µg/ml 6-thioguanine)-containing medium was initiated 24 hours after seeding. The analog-containing medium was replaced four times during the two-week selection interim.
6. One week after seeding, flasks for CFE determination were fixed and stained.
7. Two weeks after treatment with 6-thioguanine was initiated, selection flasks were fixed and stained for counting of mutant colonies.

Municipal Water Contamination Samples

Samples of contamination obtained by filtration or evaporation from municipal supplies were tested for their cytotoxicity in I-407 cultures. Before using, each sample was exposed to ultraviolet light for two hours. Aliquots of these samples were weighed in preweighed culture medium and assayed for cytotoxicity as described in Section 5. Ultraviolet light-irradiated amosite at an LD 50 concentration of 2.5 x 10^{-3} per cent served as a control for these experiments.

SECTION 5

EXPERIMENTAL PROCEDURES

Cytotoxicity Assays

The cytotoxicity of asbestos samples was quantified by measuring the inhibition of I-407 and ARL-6 colony formation following exposure to asbestos. For this assay, 10 cells/cm^2 I-407 cells or 20 cells/cm^2 ARL-6 cells were inoculated into 25cm^2 culture flasks. Twenty-four hours after the cells were seeded, the medium was replaced with medium containing asbestos. Following three days exposure to asbestos, the cells were washed twice and reincubated in asbestos-free medium. Between one and two weeks after the initiation of treatment, the cells were fixed in ten per cent formalin and stained with Giemsa for the determination of colony formation.

Leaching of U.I.C.C. Asbestos

Approximately 50 mg samples of UICC asbestos (chrysotile, amosite or crocidolite) were weighed in a pre-weighed aliquot of sterile deionized water or 5N HCl. Each weighed sample was further diluted to 0.5 per cent (w/v), stirred thoroughly to disperse the fibers throughout the fluid phase, and leached for one 96-hour or three consecutive 24-hour intervals at 25°C.

Following leaching, the asbestos samples were pelleted by centrifugation at 10,000 x g for 15 minutes, and the supernatants (except approximately 0.5 ml directly above the pellet) were removed. The asbestos pellets were resuspended in the remaining fluid and adjusted to the proper concentrations for quantification of asbestos toxicity. When three consecutive leachings were performed, each leaching was followed by centrifugation and resuspension of the asbestos pellet in sterile water or 5N HCl after the first two leachings and in culture medium after the final leaching. As a control, unleached asbestos samples were weighed in a pre-weighed aliquot of culture medium and adjusted to concentrations comparable to the leached asbestos.

Following this protocol, no significant loss of asbestos weight occurred during leaching. This was established in

control experiments in which weighed samples of asbestos were leached for one 96-hour or three consecutive 24-hour intervals, dried at 50-75°C and reweighed in a known weight of water.

Mutagenesis Experiments

ARL-6 cells were exposed to asbestos and then assayed for mutants at the HGPRT locus. Cells were seeded in 75cm^2 flasks at 16 and 32 cells/cm^2. Twenty-four hours later, the medium in the flasks seeded at 32 cells/cm^2 was replaced with fresh medium containing asbestos and the medium in the flasks seeded at 16 cells/cm^2 was replaced with fresh asbestos-free medium. Following six days of exposure to asbestos, control and asbestos-treated cultures were washed two times and assayed for mutagenesis (see Section 4).

SECTION 6

RESULTS AND DISCUSSION

Cytotoxicity of UICC Asbestos

When added to macrophages *in vitro*, asbestos fibers damage plasma and lysosomal membranes and cause cell death (1, 2, 27). This form of short-term cytotoxicity, as well as hemolysis (10, 15, 16, 34) occurs more readily in the presence of chrysotile than the amphiboles, amosite and crocidolite; however, long-term (delayed) cytotoxicity is equally induced by the serpentine and amphibole asbestos forms (20, 41, 49-51). In order to study the cytotoxic effects of chrysotile and the amphiboles and to further our understanding of the differences between the short-term and long-term effects of these fibers, cytotoxicity assays were performed on fresh samples of asbestos using ARL-6, I-407 and MCE-1 cells. The results were compared with those obtained from assays of asbestos leached in water or hydrochloric acid.

The colony forming efficiencies (CFE's) of untreated ARL-6 and I-407 cells were 50 per cent and 40 per cent, respectively; exposure of these cells to chrysotile, amosite, or crocidolite, after attachment, resulted in an inhibition of colony formation that was dependent upon dose and duration of treatment (see Figures 1 and 2). The order of toxicity was chrysotile > amosite $\simeq$ crocidolite. At least five-fold higher concentrations were required to produce a comparable inhibition of colony formation in the ARL-6 line as in the I-407 line. Although periods of exposure to the asbestos fibers were extended to include seven days, cumulative data indicated that three days' exposure produced reliable and consistent toxic inhibition. For example, after three days' treatment with 2.5×10^{-4}% chrysotile, the CFE was reduced to 56% ± 16 of that in control cultures in three repeated experiments.

The CFE of untreated mouse colon-derived MCE-1 cells was 60 to 75 per cent. When exposed to asbestos and then assayed for colony forming inhibition, the MCE-1 cells exhibited a level of susceptability to all fiber types similar to that of I-407 cells (see Figure 3). This may indicate a general sensitivity to asbestos for cells of intestinal origin.

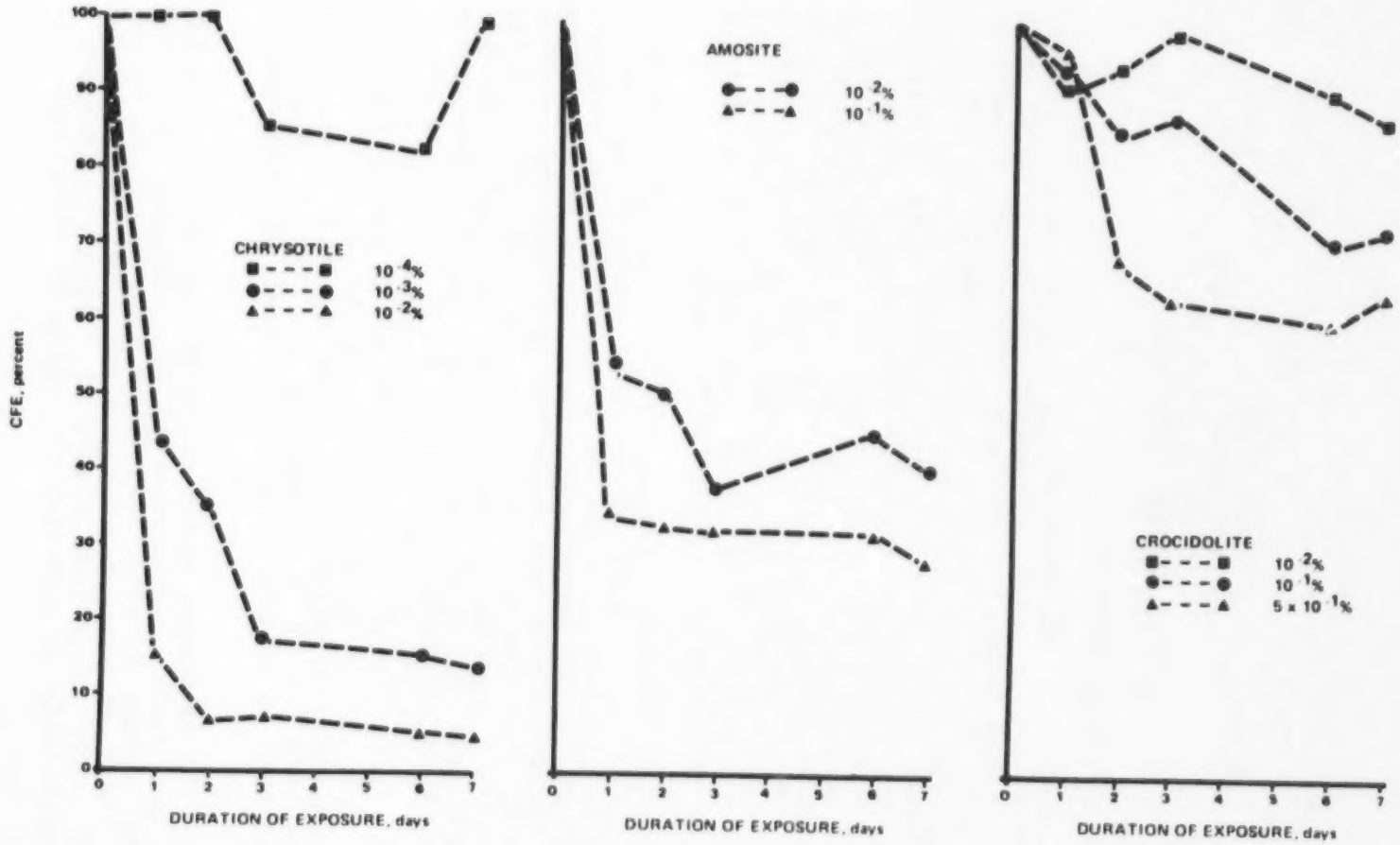

Figure 1. The cytotoxicity of chrysotile, amosite and crocidolite in ARL-6 cells

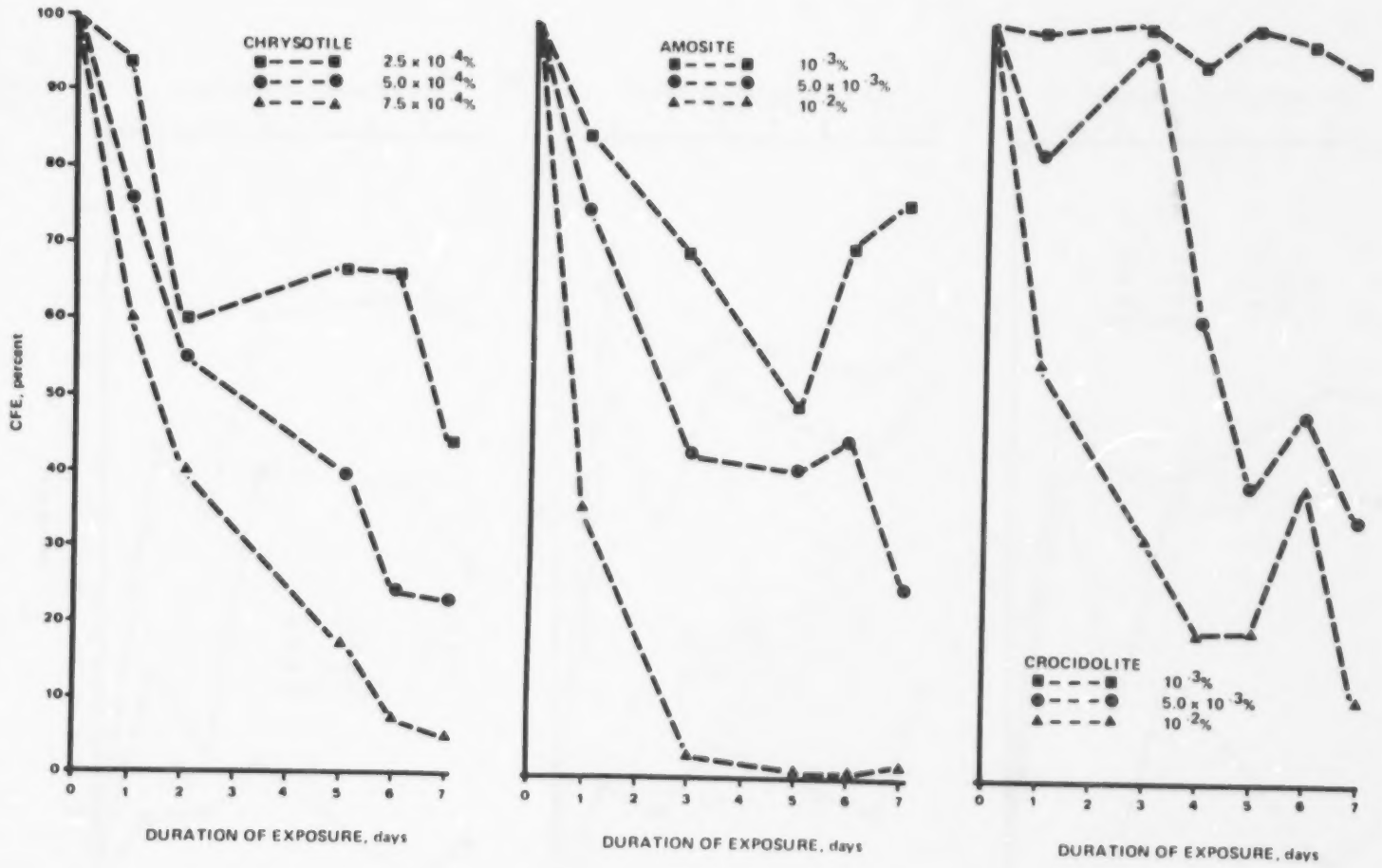

Figure 2. The cytotoxicity of chrysotile, amosite and crocidolite in I-407 cells

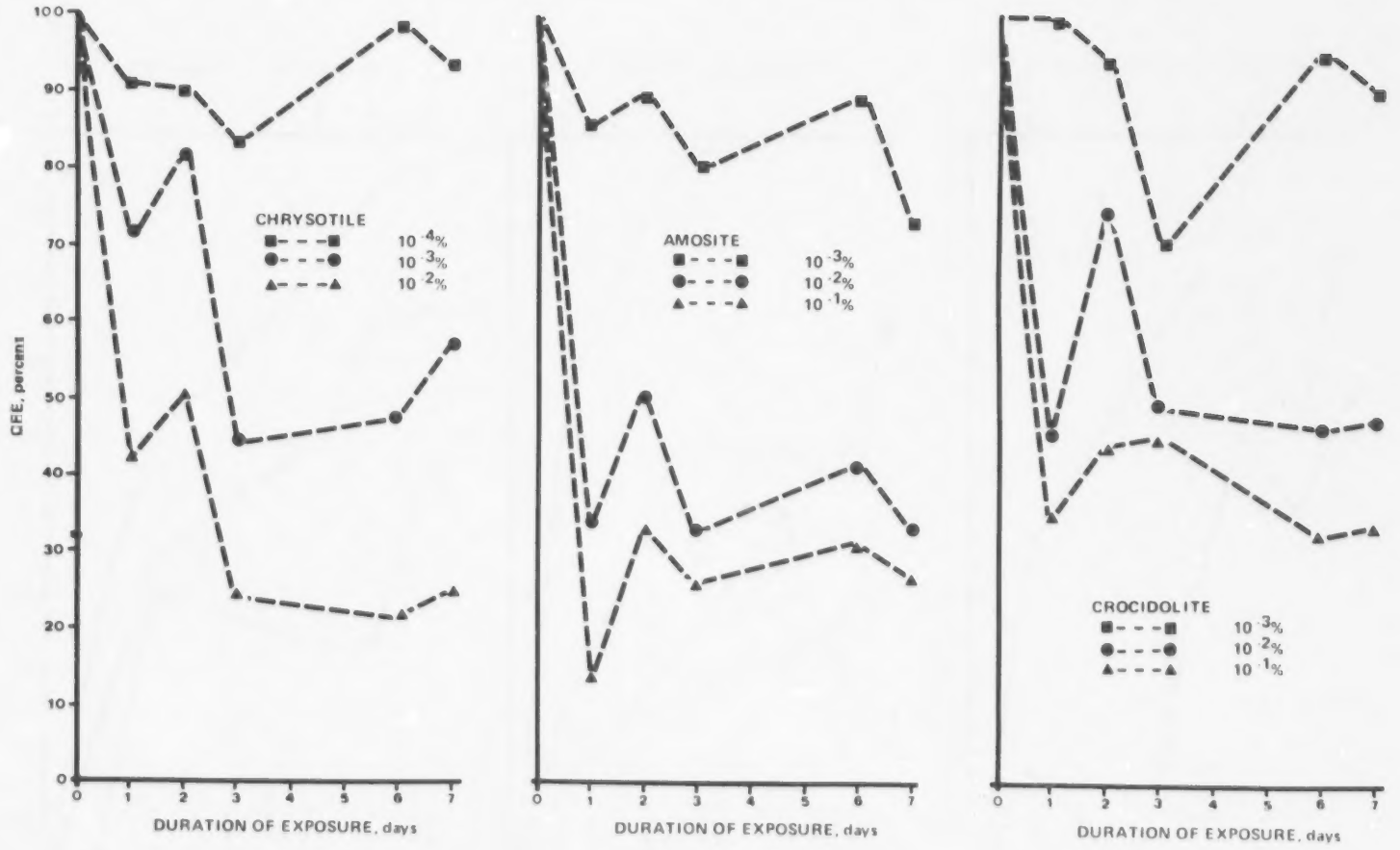

Figure 3. The cytotoxicity of chrysotile, amosite and crocidolite in MCE-1 cells

To examine the mechanism of inhibition of colony formation by asbestos, studies were performed to insure that the toxicity results were not affected by an alteration in the surface of the culture flasks produced by interaction with the asbestos fibers. In these experiments, flasks without cells were exposed to 2.5 x 10^{-4}% chrysotile in Medium E and incubated for three days at 37°C. Following incubation, the flasks were alternately scraped with a rubber policeman and washed twice and then washed again to remove all fibers before seeding with ten cells per cm^2. No significant difference was found in the CFE of cells grown in asbestos-pretreated flasks compared to the CFE of those grown in untreated flasks, indicating that inhibition of colony formation by asbestos was due entirely to a toxic effect on the cells and not an alteration of the culture surface.

In order to determine whether inhibition of colony formation was due to asbestos fibers remaining in the flasks following the two changes of medium routinely used to wash out asbestos after treatment, the toxicity of a third change of medium was examined. Colony formation of I-407 cells maintained for three or seven days in the third wash from chrysotile, crocidolite or amosite-treated I-407 cells was not supressed, indicating that the third wash did not contain levels of asbestos high enough to alter the results in this assay system (see Table 1).

Our results correspond with the data of other investigators indicating chrysotile to be more inhibitory than the amphiboles to epithelial cell and macrophage growth (2, 29, 49) and epithelial cell colony formation (29). Chamberlain and Brown (5), however, using the cloning efficiency of Chinese hamster lung cells as a criterion, found the order of asbestos cytotoxicity to be amosite > crocidolite > chrysotile. In contrast, they found that chrysotile was more inhibitory than crocidolite to human alveolar lung cell growth; growth inhibition in the presence of amosite was not determined by this group. A possible explanation for the reversed order of toxicity obtained by Chamberlain and Brown in their cloning assay might be the simultaneous addition of cells and asbestos fibers to the culture dishes (in some cases there was a 2 1/2 hour delay between seeding and addition of the fibers). We have found that the presence of fibers on the surface of a flask is inhibitory to cell attachment; furthermore, the order of adherence of asbestos fibers to the culture surface is amosite > crocidolite > chrysotile. Thus it would appear that the values obtained by Chamberlain and Brown for cloning efficiency are actually values for attachment efficiency in the presence of asbestos fibers and do not represent true asbestos cytotoxicity. The cytotoxicity of amphiboles (crocidolite, amosite and anthophyllite) contain-

TABLE 1

EFFECT ON I-407 COLONY FORMATION OF MEDIUM HARVESTED FROM TWICE-WASHED ASBESTOS-TREATED CULTURES[a]

Experiment	Harvested Medium	Days of Exposure to Harvested Medium[b] 3	7
1	Control	34	42
	Chrysotile	39	42
2	Control	95	115
	Crocidolite	98	118
3	Control	243	255
	Amosite	255	233

a I-407 cells were seeded at 5-10 cells/cm^2; 24 hrs later, the nutrient medium was replaced with medium harvested from unexposed (control) I-407 cultures or cultures exposed to 2.5×10^{-4}% (w/v) chrysotile, 5×10^{-3}% (w/v) crocidolite or 2.5×10^{-3} (w/v) amosite for 3 days. After 3 or 7 days, the harvested medium was removed from the flasks and the cells were washed twice before adding fresh medium. Colonies were allowed to develop for one week before quantification.

b Values represent the average number of colonies per flask in each group.

ing decreasing percentages of long fibers were also compared by these workers, using the same methodology (4). Decreased colony number due to inhibition of cell attachment and cytotoxicity was largely dependent upon the presence of fibers of at least 6.5 μm in length.

Neugut *et al.* (29) recently assayed two epithelial cell lines for growth inhibition in the presence of asbestos. They found, particularly in cultures of Chinese hamster

ovary, an "escape" from chrysotile toxicity after five to six days of exposure. Re-exposure to chrysotile, however, did not reveal the presence of a subpopulation of cells resistant to asbestos. Our cytotoxicity results do not indicate an escape from toxicity even 7 days after the initiation of exposure. Furthermore, these workers found that cells exposed to chrysotile for one to two days, but not longer, and then trypsinized and reseeded at cloning density in chrysotile-free medium exhibited a decreased cloning efficiency. We have observed a decreased cloning efficiency even after 6 days of exposure when cells were trypsinized and reseeded in the absence of chrysotile.

Cytotoxicity of Leached U.I.C.C. Asbestos

Samples of asbestos were leached in sterile deionized water. The cytotoxic effects of water-leached chrysotile, amosite and crocidolite were not significantly different from unleached samples in ARL-6 cells (see Table 2) or I-407 cells (see Table 3). In a further attempt to diminish the cytotoxicity of asbestos, samples were leached in 5N HCl for three consecutive 24-hour or one 96-hour interval. Unleached and water-leached chrysotile were significantly more toxic than chrysotile leached with 5N HCl; however, 5N HCl-leached amphiboles were slightly more toxic than water-leached amphiboles.

Despite the fact that water-leaching did not produce a distinct change in the cytotoxicity of the three types of asbestos, atomic absorption spectrophotometry indicated that Mg^{++} and Ca^{++} were released from the asbestos fibers into the supernatant (see Table 4). Thus, leaching in water did alter the chemistry of the asbestos fibers. Similar amounts of Ca^{++} were released from the amphiboles and chrysotile; however, a significantly greater titer of Mg^{++} was released from chrysotile than from either crocidolite or amosite.

Leaching in 5N HCl for a 24-hour interval removed more Mg^{++} from all three asbestos types, particularly chrysotile. Ca^{++} removal was also increased in 5N HCl, especially from crocidolite. A longer interval (96 hours) of leaching in 5N HCl further increased the removal of Mg^{++} but not Ca^{++} from all asbestos types.

These results suggest a direct relationship between excessive Mg^{++} and Ca^{++} depletion of chrysotile and loss of cytotoxicity and an inverse relationship between Mg^{++} and Ca^{++} depletion of the amphiboles and loss of cytotoxicity. Thus, with prolonged dispersion of asbestos fibers in a fluid environment, the cytotoxicity of chrysotile decreases while the cytotoxicity of the amphiboles increases. Similarly, Light and Wei (25) have found that leaching progressively

TABLE 2

ASBESTOS TOXICITY IN CULTURES OF ARL-6 CELLS FOLLOWING LEACHING IN STERILE DEIONIZED WATER OR 5N HCL

Asbestos	Experiment	Concentration (% w/v)	Toxicity[a]		
			Unleached	H_2O-leached	5N HCl-leached
Amosite	1	2.5×10^{-2}	79	55	54
	2	2.5×10^{-2}	74	70	98[b]
	3	2.5×10^{-2}	25	25	42[b,c]
Crocidolite	1	2.5×10^{-2}	34	42	45
	2	2.5×10^{-2}	62	63	96[b]
	3	2.5×10^{-2}	12	19	36[b,c]
Chrysotile	1	2.5×10^{-3}	93	96	29
	2	2.5×10^{-3}	95	96	75[b]
	3	3.3×10^{-3}	51	51	9[b,c]

a Inhibition of CFE of ARL-6 cells following three days exposure to asbestos; each value is expressed as the percentage inhibition of CFE for control cultures not exposed to asbestos. In experiment 1, samples were leached in water for 3 consecutive 24-hour intervals or in 5N HCl for one 96-hour interval. In experiment 2, both water-leaching and 5N HCl-leaching were performed for three consecutive 24-hour intervals. In experiment 3, both water-leaching and 5N HCl- leaching were performed for one 96-hour interval.

Cont'd.

Table 2 (cont'd.)

b Before addition to the culture medium, these asbestos samples were washed in 200 volumes sterile deionized water and recentrifuged for 15 minutes at 10,000 X g

c 5N HCl was neutralized with 1N NaOH before addition of asbestos to the culture medium.

TABLE 3

ASBESTOS TOXICITY IN CULTURES OF I-407 CELLS FOLLOWING LEACHING IN STERILE DEIONIZED WATER OR 5N HCL

Asbestos	Experiment	Concentration (% w/v)	Toxicity[a]		
			Unleached	H_2O-leached	5N HCl-leached
Amosite	1	10^{-3}	35	35	-
	1	2.5×10^{-3}	54	56	-
	2	2.5×10^{-3}	58	47	23
	3	5×10^{-3}	97	96	99[c]
	4	3.3×10^{-3}	80	74	76[b,c]
Crocidolite	1	10^{-3}			
	1	2.5×10^{-3}	45	39	-
	2	2.5×10^{-3}	23	27	16[b]
	3	5.0×10^{-3}	97	88	92
	4	3.3×10^{-3}	62	60	66[b,c]
Chrysotile	1	10^{-4}	24	40	-
	1	2.5×10^{-4}	31	39	-
	2	2.5×10^{-4}	25	35	5[b]

Cont'd.

TABLE 3 (Cont'd.)

Asbestos	Experiment	Concentration (% w/v)	Toxicity[a]		
			Unleached	H_2O-leached	5N HCl-leached
	3	5×10^{-4}	91	94	48[c]
	4	5×10^{-4}	87	71	31[b,c]

a Inhibition of CFE I-407 cells following three days exposure to asbestos; each value is expressed as the percentage inhibition of CFE for control cultures not exposed to asbestos. All samples were leached for 3 consecutive 24-hour intervals except in experiment 2 where one 96-hour interval of 5N HCl-leaching was used and experiment 4 where one 96-hour interval of water and 5N HCl-leaching were used.

b Before addition to the culture medium, these asbestos samples were washed in 200 volumes sterile deionized water and recentrifuged for 15 minutes at 10,000 x g.

c 5N HCl was neutralized with 1N NaOH before addition of asbestos to the culture medium.

TABLE 4

RELEASE OF Mg++ AND Ca++ INTO STERILE DEIONIZED WATER OR 5N HYDROCHLORIC ACID BY ASBESTOS[a]

Asbestos	Experiment	Leaching Fluid	Mg^{++}		Ca^{++}	
			Unfiltered	Filtered[b]	Unfiltered	Filtered[b]
Amosite	1	sterile deionized water	-	34.9	61.7	107.0
	2	sterile deionized water	22.4	92.3	94.7	90.5
	3	5N hydrochloric acid	394.0	-	119.3	
	4	5N hydrochloric acid	473.8	546.0	90.5	205.8
Crocidolite	1	sterile deionized water	10.0	27.4	102.9	107.0
	2	sterile deionized water	10.0	59.9	144.0	86.4
	3	5N hydrochloric acid	221.9	-	1,172.8	-
	4	5N hydrochloric acid	281.8	344.0	806.6	2,448.6

Cont'd.

TABLE 4 (Cont'd.)

Asbestos	Experiment	Leaching Fluid	Mg^{++} Unfiltered	Mg^{++} Filtered[b]	Ca^{++} Unfiltered	Ca^{++} Filtered[b]
Chrysotile	1	sterile deionized water	548.6	498.8	37.0	205.8
	2	sterile deionized water	498.8	1,034.9	37.0	234.6
	3	5N hydrochloric acid	20,448.9	-	49.4	-
	4	5N hydrochloric acid	34,663.3	33,915.2	65.8	181.1
None	1	sterile deionized water	0.0	2.5	0.0	16.5
	2	sterile deionized water	0.0	0.0	0.0	0.0
	3	5N hydrochloric acid	0.0	-	0.0	-
	4	5N hydrochloric acid	0.0	-	0.0	-

Cont'd.

Table 4 (Cont'd.)

a Values for the concentrations of Mg^{++} and Ca^{++} were obtained in parts per million (ppm) by atomic absorption spectrophotometric analysis of leaching-fluid samples following 24 hours (experiments 1,2 and 3) or 96 hours (experiment 4) of leaching at 25°C, at 0.5 per cent (w/v). Each sample was monitored using reference standards of known Mg^{++} or Ca^{++} ppm and then converted to μ moles by division with 0.0401 (for Mg^{++} values) or 0.0243 (for Ca^{++} values).

b Leachate filtered through a Nalgene 0.2μ (experiments 1 and 3) or 0.45μ (experiments 2 and 4) filter unit before atomic absorption spectrophotometric analysis.

decreases the surface charge and hemolytic activity of chrysotile but increases these properties for the amphiboles. Such convergence of activities would explain the equal effects of the serpentine and amphibole forms of asbestos in delayed cytotoxicity, following a prolonged interval of *in vivo* leaching. These results correspond with those of Pelfrene (31) who also noticed a preferential Mg++ leakage from chrysotile and a preferental Ca++ leakage from the amphiboles, particularly crocidolite, during saline leaching.

Mutagenicity of UICC Asbestos

Chrysotile and crocidolite have been reported to induce chromosome damage in cultured Syrian hamster embryo cells (23), Chinese hamster ovary cells (38), murine 3T3 cells (37) and Chinese hamster lung cells (17). Although no mutagenic activity was found to be associated with amosite, crocidolite or chrysotile in mutation tests using E. coli or S. typhimurium (6), crocidolite was reported to have weak mutagenic activity at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultures of Chinese hamster lung cells (17). Therefore, in order to develop further information on the possible genetic effects of asbestos, the three forms of asbestos were examined for mutagenicity using the HGPRT mutagenesis assay on ARL-6 cells, developed in this laboratory.

Exposure to chrysotile at concentrations of 7.5×10^{-4} and 10^{-3} per cent , resulted in a statisically significant greater mutant recovery than was obtained in nonexposed cultures in one out of five experiments (Table 5,a). Crocidolite at 10^{-2} and 2×10^{-2} per cent yielded a greater mutant recovery in three out of seven experiments (Table 5,b). Amosite exposure did not produce an increased mutant recovery in two experiments (Table 5,c). Although the slight increase in mutant recovery after exposure to high concentrations of asbestos was in some cases statisically significant, the results of these experiments cannot be taken as evidence of mutagenicity because the mutant incidences after asbestos exposure in all but one experiment did not exceed the range of incidences (i.e., up to 17.3 mutants per 10^6 colony forming cells) observed in control experiments. Furthermore, the 98% confidence level for increased incidences in this line has been established to be 11.8 ± 3.7 mutants per 10^6 colony forming units (47), and none of the incidences in exposed cultures significantly exceeded this value. Thus, the results indicating increased mutant recovery following exposure to asbestos must be considered to be due to fluctuations in spontaneous mutant recovery between experiments. Therefore, we conclude that in this series of experiments, asbestos was not mutagenic.

Table 5

Asbestos-Induced Mutagenesis at the HGPRT Locus in ARL-6 Cells[a]

Asbestos	Concentration (% w/v)	Mutants/10^6 colony forming units: Asbestos-Exposed	Control	P Value[b]
a. chrysotile	7.5×10^{-4}	4.2 ± 4.6	0	N.S.
	10^{-3}	26.0 ± 20.7[c]	0	<0.025
	10^{-3}	6.7 ± 11.3	0	N.S.
	7.5×10^{-4}d	0	0	N.S.
	10^{-3}d	0	0	N.S.
b. crocidolite	10^{-2}	7.5 ± 4.7	0	<0.005
	2.0×10^{-2}	2.0 ± 4.9	0	N.S.
	10^{-2}d	0	0	N.S.
	2.0×10^{-2}d	0	0	N.S.
	10^{-2}	10.0 ± 12.7	17.3 ± 12.1	N.S.
	2.0×10^{-2}	0	17.3 ± 12.1	<0.01
	3.0×10^{-2}	0	17.3 ± 12.1	<0.01
c. amosite	10^{-2}	0	0	N.S.
	2.0×10^{-2}d	5.9 ± 14.6	0	N.S.

a This table represents the results of fourteen experiments. For these experiments, cells were exposed to asbestos for six days as described in Section 5 and assayed for mutagenesis as described in Section 4. Preliminary experiments performed before our selection technique was refined showed high spontaneous mutant incidences; these results are not presented.

b Significance was determined by means of the Student's t-test.

Cont'd.

Table 5 (Cont'd.)

c This value exceeded 11.8 ± 3.7, the 98% confidence limit for increased incidences with $p < 0.1$.

d The cells used in this experiment were non-transformed ARL-6 cells.

These results are in agreement with bacterial mutation tests demonstrating no mutagenicity for amosite, crocidolite or chrysotile (6). They do not differ greatly from the weak mutagenicity results obtained for crocidolite at the HGPRT locus in Chinese hamster lung cells (17); in this study, a slight increase in mutant incidence was observed in only one experiment. Since the range of variation in spontaneous mutant recovery was not presented, it cannot be determined whether this result, unlike the present results, was outside the 98% confidence limit for spontaneous mutant incidence.

Although it has been reported that asbestos fibers produce major chromosome breaks and aberrations in Syrian hamster cells (23), Chinese hamster ovary cells (38) and murine 3T3 cells (37), these breaks are dependent upon the presence of a specific size range of fibers, either asbestos or glass, and are not necessarily related to the production of point mutations.

Cytotoxicity of Municipal Water Contamination

Six samples, provided by the EPA, were tested for cytotoxicity in I-407 cultures. Before testing, all samples were irradiated with ultraviolet light to elimate contamination with micro-organisms. Amosite, at an LD 50 concentration of 2.5×10^{-3} per cent, served as a positive control. The results (see Table 6) demonstrated that, by assaying the inhibition of I-407 cell colony formation, the cytotoxic levels of the six samples could be easily evaluated. The order of toxicity of these samples, as determined by the cytotoxicity assay, was: sample no. $8>3>2>1\gtrsim5>4$.

Project Officer's Note

Samples of particulates, some extracted directly from drinking waters were sent to the grantee referenced only by a code number. The cytotoxicity testing was then done "blind" using the I-407 cultures. Sample No. 8 was a sample of the amosite fibers currently being used to study the effects of ingested asbestos on rats and hamsters by the National Institute of Environmental Health Sciences (NIEHS). The feeding study is partially supported by the Environmental Protection Agency. Not surprisingly the cytotoxicity of the NIEHS amosite was very similar to that of the UICC amosite used as a positive control. Sample Nos. 3,2, and 1 were particulates collected by filtration from drinking waters from San Francisco, Seattle, and Duluth (prior to the installation of the filtration plant at Duluth) respectively. Chrysotile fibers have been identified in the particulate samples from San Francisco and Seattle; amphibole fibers were identified among the particulates from the Duluth sample. Sample No. 5 was a sample of attapulgite clay, a non-asbestos mineral, which consisted of fibers in the same range as chrysotile asbestos. Attapulgite fibers have been identified in some

water supplies in Georgia and Florida. Sample No. 4 was a sample of the less than 2 micrometer size fraction of taconite tailings which had been prepared by a sedimentation separation procedure. Amphibole fibers were identified among the particulates in sample No. 5.

All the particulate samples were less cytotoxic than the commercial asbestos variety samples. Details concerning the characteristics of the particulate in the various samples and further discussion of the differences in cytotoxicity will be forthcoming in a subsequent paper.

TABLE 6

TOXICITY TO THE I-407 CELL LINE OF SAMPLES OF CONTAMINATION FROM MUNICIPAL WATER SUPPLIES

Concentration of Sample (% w/v)	Percent Inhibition of Colony Formation [a] Sample Number 1	2	3	4	5	8
10^{-4}	-	-	-	-	8	18
2.5×10^{-4}	-	-	-	-	-	-
5.0×10^{-4}	21	-	-	-	9	5
7.5×10^{-4}	-	-	-	-	-	-
10^{-3}	-	10	-	1	16	54 ± 13[b]
2.5×10^{-3}	21	-	-	0	-	72 ± 12
5.0×10^{-3}	0	-	-	1	-	86
7.5×10^{-3}	-	16	-	-	-	-
10^{-2}	-	9	-	-	9	-
2.5×10^{-2}	-	-	6	3	-	-
5.0×10^{-2}	6	-	-	-	5	-
7.5×10^{-2}	-	49	6	-	-	-
10^{-1}	1	37[b]	96	2	3	-
2.5×10^{-1}	30	73	100	33	35	-
5.0×10^{-1}	88[b]	91	-	41	43	-

a These values represent the results of a total of seven experiments; many of these values are averages from duplicate experiments. Where more than two experiments were averaged, the standard deviation is included. Each value is the per cent inhibition of CFE of I-407 cells following exposure to water contamination samples. The average CFE for all experiments of I-407 cells not exposed to water contamination samples or amosite was 112.5% ± 41.2. An amosite standard at

(Cont'd)

TABLE 6 (Cont'd)

2.5×10^{-3}% (w/v) was tested simultaneously with each experiment; the average per cent inhibition of CFE for all experiments of amosite-exposed cells was 59.2 ± 15.1. Chrysotile at 2.5×10^{-4}% (w/v) exhibited approximately a 50% inhibition of CFE in I-407 cell cultures.

b A result widely divergent from this value was omitted because it was inconsistant with the trend of increasing toxicity with increasing concentration of the sample.

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